Expression and Characterization of MMP1090 UDP-Gal/UDP-GalNAc Epimerase and Investigation into UDP-Sugar Assays

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Abstract:

A gene found in *Methanococcus maripaludis*, MMP1090, is a homolog of known UDP-glucose (UDP-Glc)/UDP-galactose (UDP-Gal) and UDP-N-acetylglucosamine (UDP-GlcNAc)/UDP-N-acetylgalactosamine (UDP-GalNAc) 4-epimerases. These UDP-sugars are integral in the biosynthetic pathways of many organisms, from sugar metabolism in humans to cell wall formation in *Escherichia coli.* The MMP1090 epimerase is expected to function through abstracting a hydrogen on the C4 carbon of the UDP-sugar and inducing a rotation about the phosphorus-oxygen bond connecting the UDP to the sugar. This enzyme was successfully expressed and purified; subsequently, the protein successfully epimerized UDP-Glc and UDP-GlcNAc to UDP-Gal and UDP-GalNAc, and kinetic parameters were determined. Utilizing this epimerase, the possibility of a coupled-concentration assay for UDP-Gal using *E. coli* as the test organism was explored, where the assay reaction involves epimerization of UDP-Gal to UDP-Glc followed by oxidation by a dehydrogenase and reduction of NAD^+ which can be detected by fluorescence. By using an optimized UDP-Glc concentration assay, growth conditions that could affect UDP-Glc levels in *E.* coli were investigated. Glucose supplemented LB-medium was shown to cause significantly elevated UDP-Glc in *E. coli*—signifying increased sugar metabolism—compared to *E. coli* grown in normal LB-medium. UDP-Glc levels in *E. coli* grown in aerobic conditions appear to show no significant difference when compared with *E. coli* grown in semi-anaerobic conditions.

Introduction:

Gene sequence analysis showed a gene, MMP1090, isolated from *Methanococcus maripaludis* to be a possible homolog of known UDP-glucose and UDP-N-acetylglucosamine 4-epimerases, which catalyze the reversible epimerization of UDP-glucose (UDP-Glc) and UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-galactose (UDP-Gal) and UDP-N-acetylgalactosamine (UDP-GalNAc)[1]. *M. maripaludis* (from the Latin "mare" meaning sea and "palus" meaning marsh) is a mesophilic methanogen discovered in a salt-marsh in North Carolina that has been a model organism among methanogenic Archaea[2]. Comparative gene sequence analysis and the presence of this epimerase along with other sugar metabolizing enzymes suggest that this organism follows a sugar biosynthetic pathway similar to that of bacteria[3]. Investigation into these enzymes and pathways can give insight to the different sugars involved and their roles in cellular function. The enzymes and the reactions they are involved in can be used as tools to assay the substrates they act on.

The MMP1090 epimerase acts to convert UDP-Glc to UDP-Gal and UDP-GlcNAc to UDP-GalNAc, all activated forms of their respective sugars. Each of these activated sugars has a myriad of roles in organisms. In *E. coli,* for example, UDP-Glc and UDP-Gal are substrates in the biosynthesis of lipopolysaccharides and the capsular colonic acid that coats the bacteria[4]. UDP-Glc also appears to serve a role as a potential intracellular signal for gene regulation[4]. UDP-GlcNAc and UDP-GalNAc has a multi-role function across all three domains of life which includes use in cell walls, extracellular matrices, and protein post-translational modifications such as glycosylation, and glycolipids[5]. Methanogens such as *M. maripaludis* have been hypothesized to use specifically UDP-GalNAc and UDP-GlcNAc as precursors for flagellin, S-layer proteins, and coenzyme B[2].

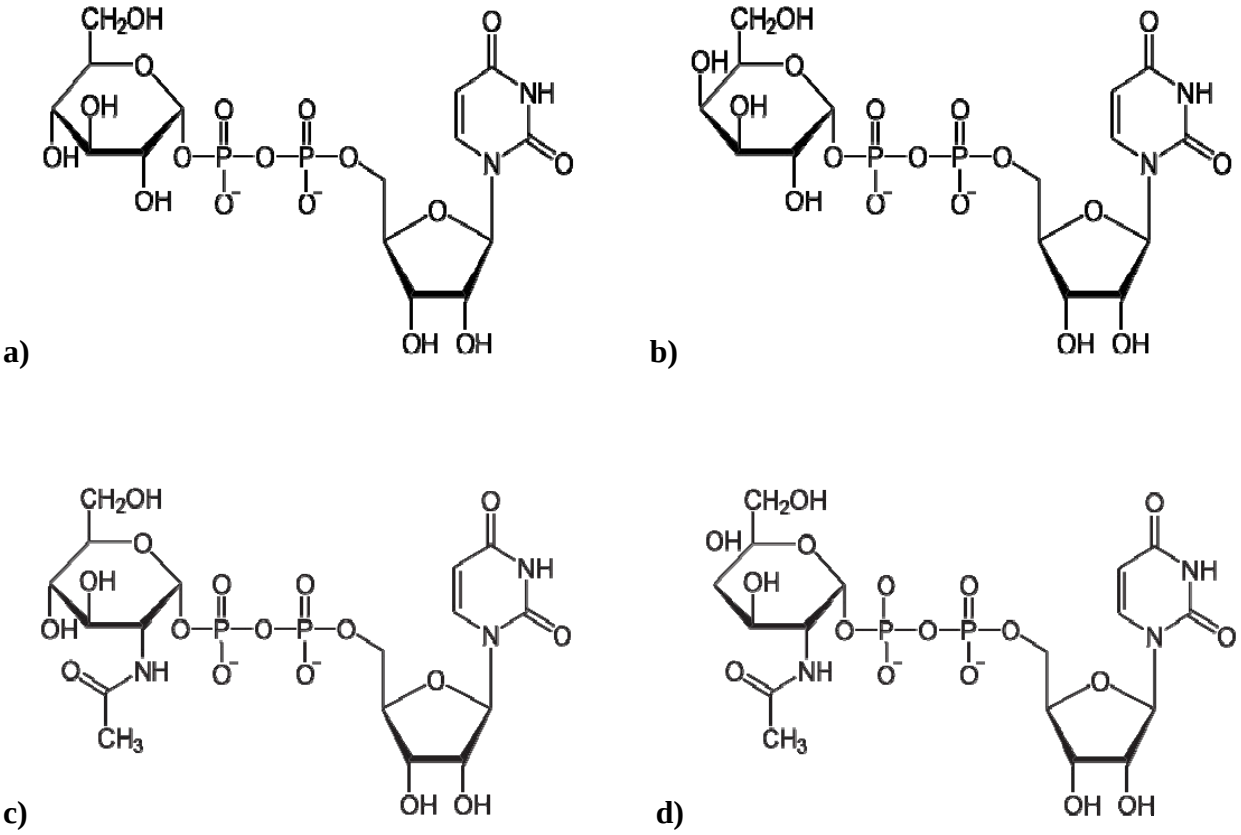

Figure 1: These represent the molecular structures of the different UDP-sugars. a) UDP-Glucose; b) UDP-Galactose; c) UDP-N-acetylglucosamine; d) UDP-N-acetylgalactosamine.

The mechanism of this enzyme for UDP-Gal/UDP-Glc epimerization in other organisms has been highly researched. One possible mechanism in *E. coli* UDP-Gal 4-epimerase is discussed by Thoden, *et al*[6]. In the proposed mechanism, an enzymatic base on the enzyme first abstracts a C-4 hydroxyl hydrogen on the UDP-sugar. The hydride abstracted is transferred to a tightly bound NAD^+ molecule on the enzyme, resulting in a 4-ketopyranose intermediate and a reduced NADH cofactor. A reversible conformational change occurs in which there is a rotation about the phosphorus-oxygen bond connecting the UDP to the sugar. The rotation about the bond allows the return of the hydride from the NADH to the opposite face of the sugar, completing the epimerization. This mechanism is summarized in Figure 2 below. Analysis of mutations of the epimerase has identified Lysine 153 in the protein sequence promotes binding

of NAD^+ at the coenzyme site through hydrogen bonding between the 2' and 3' hydroxyl groups on the nicotinamide riboside and the ammonium group on lysine[7]. In humans, the UDP-Gal/UDP-Glc epimerase is an essential enzyme in metabolizing galactose. A deficiency in the enzyme may cause the rare-genetic disorder galactosemia, which can result in toxic levels of galactose that can lead to serious medical complications such as renal failure, cirrhosis, and brain damage[17]. The epimerase of *M. maripaludis* is expected to occur through a similar mechanism as described above in both UDP-Gal/UDP-Glc and UDP-GalNAc /UDP-GlcNAc epimerization.

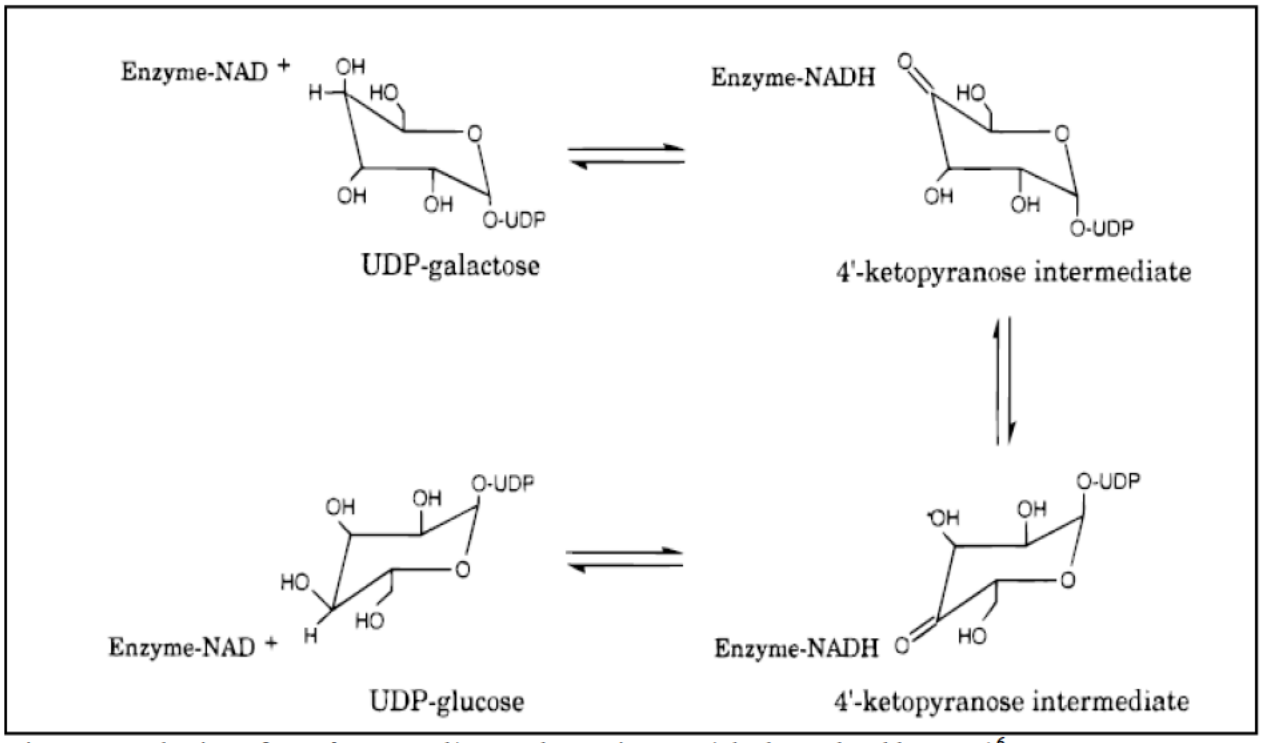

Figure 2: Mechanism of *E. coli* UDP-Gal/UDP-Glc 4-Epimerase (Thoden and Holden 1998)[6]

The MMP1090 gene was isolated, inserted into a plasmid vector, and introduced into *E. coli* BL21(DE3) for protein expression and purification courtesy of Dr. David Graham. The purification process encompasses nickel-affinity chromatography as the expressed MMP1090 gene contains a terminal 6-Histidine tag that can selectively bind nickel. Once purified, the enzyme was characterized and kinetics parameters were determined using both UDP-Gal and UDP-GalNAc as substrates. Activity was assayed through measuring the formation of the UDP-

sugar's respective epimers with ion-exchange chromatography. Due to the differences in their molecular structures, the UDP-sugars can be identified as they elute at different times. Kinetic parameters were determined through coupling the epimerase with a dehydrogenase that oxidizes the epimerase product and reduces NAD^+ to NADH. The NADH formation can be measured through UV-Vis Spectrophotometer as NADH absorbs UV light at 340 nm.

A UDP-Gal concentration assay was created using the epimerase in combination with a dehydrogenase enzyme through a coupled reaction. The assay used a standard addition method where an extract of a sample organism such as *E. coli* was assayed along with standard concentrations of UDP-Gal. The concentration determined was the total concentration of both UDP-Gal and UDP-Glc as the dehydrogenase also oxidized any available UDP-Glc. An optimized UDP-Glc assay (per Dalia Carranza) was used to determine UDP-Glc concentration, and the difference between the two assays gave the total UDP-Gal concentration in the cell.

The UDP-Glc assay was also used to test different growth conditions of *E. coli* that may affect UDP-Glc levels. One experiment evaluated the UDP-Glc levels of *E. coli* grown in grown in regular LB-medium compared with *E. coli* in glucose-supplemented LB-medium. Being grown in a glucose rich environment, the *E. coli* cells was expected to increase their glucose metabolism as the reaction is pushed forward based on Le Chatelier's Principle (i.e. glucose is metabolized to reach the equilibrium state). As glucose metabolism increases, the UDP-Glc levels were expected to increase since this activated sugar plays an important role in sugar metabolism and biosynthesis. The effects of aerobic growth vs. semi-anaerobic (i.e. fermentation) growth on UDP-Glc levels was also tested. UDP-Glc levels were expected to be higher in aerobic conditions as cells typically undergo higher levels of metabolism during respiration than fermentation.

Materials and Methods

Isolation and Expression of MMP1090:

MMP1090 gene transformation was performed by Dr. David Graham. The MMP1090 gene was amplified from chromosomal DNA of *M. maripaludis* by PCR using the forward primer 5MMP1090BN and the reverse primer 3MMP1090X. The PCR product was purified and digested with NdeI and XhoI restriction enzymes, and then ligated into similarly digested plasmid vector pET-43.1a to produce plasmid pDG473, which was then amplified and purified. In addition to MMP1090, the plasmid also contained an ampicillin resistance gene for screening and the lac operon for expression. The pDG473 plasmid was introduced into *E. coli* BL21(DE3), and transformed cells were selected by ampicillin. Transformed *E. coli* were then grown in LB/ Ampicillin medium (1 µL ampicillin/ 1 mL of LB-medium), incubating at 37°C and shaking at 250 rpm. Through a 1 mL sample, optical density at 600 nm was measured to determine bacteria growth. When the OD_{600} was at 0.6-0.8, lactose was added to induce protein expression through the lac operon. Time point samples at t=0 hr (before lactose addition), t=1 hr and t=3 hrs (after lactose addition) were taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to check for expression. At t=3 hrs, the cell cultures were centrifuged, the supernatant was decanted, and the pellet was resuspended in distilled water. The cells were then lysed with a sonicator and centrifuged, with the protein assumed to be in the supernatant. An SDS-PAGE was performed on the samples of the lysed cells, both supernatant and resuspended pellet, to determine whether the protein resided in a soluble or insoluble matrix.

Purification of MMP1090:

The expressed MMP1090 gene contained a poly-histidine tag at its C-terminus. With this tag, the MMP1090 protein was purified using Ni^{2+} column chromatography. Ni^{2+} coordinates

with the imidazole ring located in the histidine, which allows for binding affinity chromatography. The starting buffer used in the purification was 20 mM Tris/HCl at pH 7.6 with 500 mM NaCl, while the elution buffer contained the starting buffer in addition to 1 M imidazole to displace the MMP1090 from the column. Fractions containing MMP1090 were determined using an SDS-PAGE gel. The fractions containing the highest amount of protein were pooled together.

Characterization of MMP1090:

A Coomassie Protein Assay was performed to analyze the concentration of the pooled purified protein to allow the manipulation of protein amount in the reactions. In addition, size-exclusion chromatography was on the purified protein (per Dr. David Graham and Seema Namboori) to determine molecular mass of the native form of the enzyme. By comparing the molecular mass of the native form of the enzyme to the molecular mass based on the gene sequence using ExPASy Proteomics tools, the quaternary structure of MMP1090 was determined.

The protein was then tested for epimerase activity through observing the successful epimerization of UDP-Glc and UDP-GlcNAc to UDP-Gal and UDP-GalNAc. In the initial reaction, 20 μg of MMP1090 was combined with 50 mM KCl, 1 mM UDP-Glc/UDP-GlcNAc, 1 mM NAD^+, and 100 mM potassium phosphate buffer pH 7.0. Control reactions without MMP1090 and without the substrate (UDP-Glc/UDP-GlcNAc) were also done. The reaction was allowed to reach equilibrium overnight at 37°C. An ion-exchange column chromatography was performed on the sample to observe epimerization as glucose metabolites elute 1-2 minutes slower than galactose metabolites due to the different interactions with the column. A second set

of reactions were done with the same parameters and methods but without NAD^+. Optimization for pH was then also done with the same parameters and methods but with different pH buffers in place of the phosphate buffer: 50 mM Tris/HCl pH 8.0; 50 mM Tris/HCl pH 8.5; and 0.2 M Methoxyamine/0.5 M Glycine pH 9.5.

Determination of Kinetic Parameters:

Kinetic parameters were then determined for the MMP1090 epimerase with UDP-Gal and UDP-GalNAc as substrates by using a coupled reaction with a dehydrogenase (MMP0352 UDP-GlcNAc dehydrogenase/ MA4457 UDP-Glc dehydrogenase) that oxidizes the epimerase product (UDP-GlcNAc to UDP-GlcNAc-3-oxo and UDP-Glc to UDP-Glucuronic acid) and reduces NAD+ to NADH. The formation of NADH was detected by UV-Vis spectrophotometry (absorbance at 340 nm), and the specific activity of a reaction at different substrate concentration was evaluated through the kinetic function of the spectrophotometer.

Each substrate had a different set of optimized reaction conditions based on the dehydrogenase involved in the coupled reaction. These optimized conditions were experimentally determined for the MMP0352 UDP-GlcNAc dehydrogenase by Seema Namboori and the MA4457 UDP-Glc dehydrogenase by Dalia Carranza. The amount of epimerase added to the coupled reactions was equal to the amount of dehydrogenase. These reactions were done in a 300 µL volume. For the coupled reaction with UDP-GalNAc as the substrate, the reaction mixture contained 200 mM KCl, 1 mM NAD+, 30 µg MMP0352, 100 ng MMP1090, 100 mM N-Cyclohexyl-2-aminoethanesulfonic acid **(**CHES)/NaOH pH 9.5, and 10-800 µM of UDP-GalNAc. With UDP-Gal, the mixture contained 200 mM KCl, 1 mM NAD+, 30 µg MMP0352, 100 ng MMP1090, 100 mM CHES pH 9.5, and 10-800 µM of UDP-GalNAc. The rates of

epimerization were directly evaluated through the absorbance of NADH production by the UV-Vis spectrophotometer. After obtaining rates for the enzyme at different substrate concentrations, the data were entered into Kaleidagraph where K_m and V_{max} values were determined for the enzyme.

Development of UDP-Gal Assay

Based on the coupled reaction below,

UDP-Gal + MMP1090 ⟶ UDP-Glc + MA4457 $\xrightarrow{2NAD^+ \rightarrow 2NADH + 2H^+}$ **UDP-Glucuronic acid**

it is expected that 2NAD+ molecules are reduced for every 1 UDP-Gal molecule epimerized. This stoichiometry was verified by comparing the NADH production of samples of the optimized reaction mixture (containing 300 µM NAD+, 60 mM KCl, 5 µg MA4457, 5 µg MMP1090, 50 mM Tris pH 8.0, 5 mM DTT, with incubation for 1 hour at 37°C) with standard concentrations of UDP-Gal as substrates to standard NADH samples through NADH fluorescence with a fluorimeter (excitation wavelength of 340 nm and emission wavelength of 460 nm).

A UDP-Gal assay that utilizes the coupled reaction above and the method of standard addition was investigated. A wild strain of *E. coli* was assayed. The *E. coli* was extracted using a method modified from a method outlined in Namboori and Graham[5]. A 1% (v/v) overnight culture of wild-type *E. coli* culture B, originating from the Coli Genetic Stock Center (CGSC 5365), was grown in 100 mL LB-medium with vigorous shaking at 37°C. When OD_{600} reached approximately 0.8-1.0, the culture was split into 10 mL fractions and harvested by centrifugation at 5000 g for 10 minutes. Later when testing UDP-Glc levels in aerobic vs anaerobic conditions, the *E. coli* in anaerobic conditions will have slow growth so OD_{600nm} of 0.5 -0.7 will suffice because dry mass will be measured. The pellets were kept frozen at -20°C until needed. Each

pellet fraction was resuspended in 200 μL of 5% trichloroacetic acid (TCA) at room temperatures for 20 min. Afterwards, the sample was centrifuged at 5000 g for 10 min to remove cell debris. The supernatant was kept at room temperature for 15 min and was neutralized with 15 μL of 2.5 M KOH in 1.5 M K_2HPO_4 and was stored at -20°C until assayed. The dry masses of the pellets were determined after drying at 100°C for 2 days.

The extracted sample of *E. coli* was combined with standard concentrations of UDP-Gal, and a standard addition curve was produced (fluorescence of NADH vs. standard concentrations of UDP-Gal). This method was adapted from a UDP-GlcNAc assay by Namboori and Graham[5]. The extrapolated concentration at zero absorbance determined the total concentration of substrate oxidation. The total concentration measured was actually the sum of both UDP-Gal epimerized and UDP-Glc already present in the cell. A UDP-Glc assay (optimized by Dalia Carranza, similar to the combined UDP-Gal/UDP-Glc assay but with the absence of the epimerase) was used to measured just the UDP-Glc present in the cell extracts. The actual concentration of UDP-Gal in the cells was the difference between the two assays. Using standard addition allows for any interference that may cause inaccuracies in the fluorescence of NADH. All reactions were performed in a 1 mL mixture. For the UDP-Glc + UDP-Gal assay, the reaction mixture contained: 300 μM NAD+, 60 mM KCl, 5 μg MA4457, 5 μg MMP1090, 50 mM Tris pH 8.0, 5 mM DTT, and 15 μL *E. coli B* extract. Different samples were supplemented with 0-3 μM UDP-Gal for the purpose of standard addition, with each substrate concentration done in triplicate. Background fluorescence was accounted for with two control reactions: one contains only the buffer with *E. coli B* extract to account for extract fluorescence; the other contains the reaction mixture without any UDP-Gal or extract to account for enzyme-catalyzed NAD^+ adduct formation. For the UDP-Glc assay, the reaction mixture contained 300 μM NAD+, 60 mM KCl,

5 µg MA4457, 50 mM Tris pH 8.0, 5 mM DTT, and 15 µL *E. coli B* extract. Control reactions were also performed similarly as above, but 0-3 µM UDP-Glc was instead used for standard addition. All reaction mixtures were incubated for 1 hr at 37°C and NADH fluorescence was measured as stated above in the verification of stoichiometry. A plot of NADH fluorescence vs. substrate concentration was created, and a least-squares analysis was performed to determine the x-intercept, which gives the substrate concentration in the cell extracts. Error was propagated through knowing the error in slope and y-intercept through the MS Excel LINEST function. The concentrations determined in the cell extracts was converted to moles of the UDP-sugar per gram of *E. coli* (based on dry mass).

Different Growth Conditions of E. coli on UDP-Glc levels

Using the UDP-Glc assay described above, growth conditions that affect the UDP-Glc levels in *E. coli* was investigated. The first condition examined was the effects of glucose-supplemented growth media. *E. coli* was grown in two different set of media: one normal LB-medium (1% NaCl w/v, 0.5% yeast extract w/v, 1% Casein w/v); one glucose supplemented LB-medium (1% NaCl w/v, 0.5% yeast extract w/v, 1% Casein w/v, 1% D-glucose w/v). Both set of *E. coli* was grown by incubating at 37°C with vigorous shaking. Extraction of the cells was performed as before. The UDP-Glc assay was performed as described above on the two set of cells and the moles of UDP-Glucose per gram of *E. coli* was compared. A t-test was performed on the two data set to evaluate any significant difference between the levels of UDP-glucose in each condition.

Another set of conditions that was explored were the effects of aerobic conditions vs. anaerobic growth conditions on UDP-Glc levels. Two sets of *E. coli* cultures was grown in LB

medium at 37°C, but one was grown with vigorous shaking which grows the cells under aerobic conditions while the other culture without vigorous shaking which grows the cells under semi-anaerobic conditions (some residual oxygen may enter the culture flasks). Extraction and the UDP-Glc assay was done as above and a t-test evaluated the significance of the data.

Results

Expression and Characterization of MMP1090 Epimerase

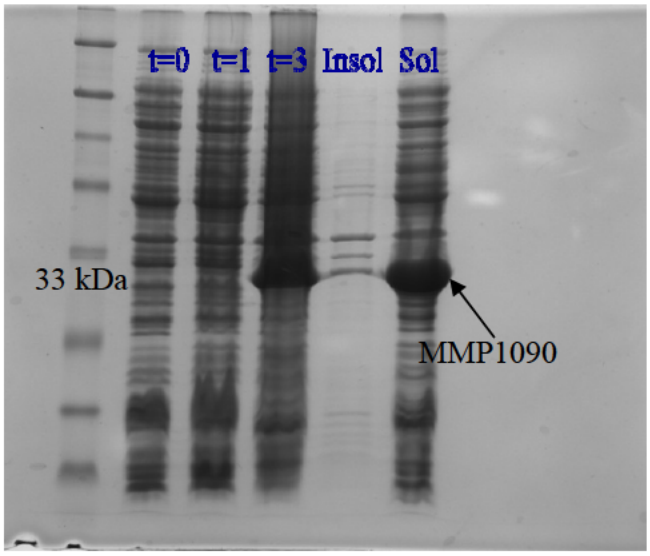

Figure 3: SDS-PAGE gel of MMP1090 expression at time points after lactose addition and of soluble and insoluble phase.

Figure 3 above shows the SDS-PAGE gel of the samples taken while expressing the protein. At ~33 kDa, there appears to be a gradual increase in the darkness and heaviness of the band. Using ExPASy proteomics tools, the MMP1090 protein sequence was determined using the gene sequence, and the molecular mass was determined using a protein molecular mass calculator. The MMP1090 epimerase was calculated to be about 33.6 kDa, roughly what is displayed on the gel. The soluble phase of the cells contains the protein while the insoluble does not.

The SDS-PAGE gel below, figure 4, displays the presence of MMP1090 in different fractions of the Ni^{2+} chromatography performed. Protein appears at 33 kDa, confirming the purification of MMP1090. The fractions marked with an X were pooled to make the stock solution of MMP1090.

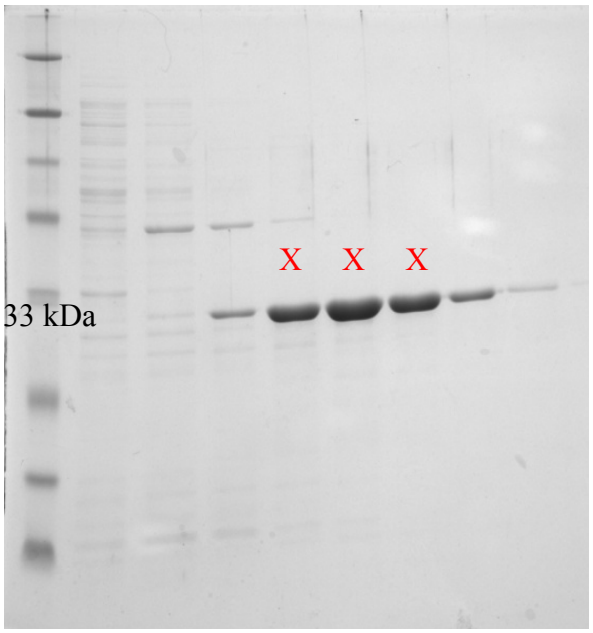

Figure 4: Expression of purification fractions

A coomassie total protein assay was performed on the pooled samples to determine the concentration of the protein solution. The absorbance of the pooled solution was compared against the standard curve, Figure 5, of the assay to calculate concentration, which was found to be 1.25 mg of MMP1090/mL.

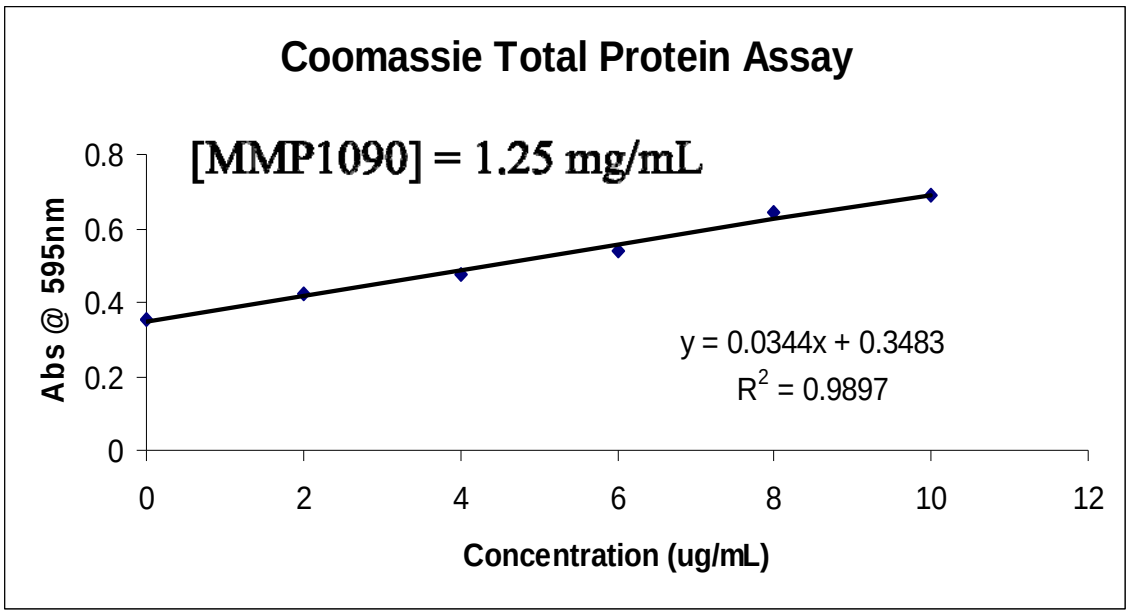

Figure 5: Standard curve of the coomassie total protein assay.

The size exclusion chromatography performed resulted in the following standard curve displayed in Figure 6. Knowing the elution volume of MMP1090 allows for the interpolation of the molecular mass of the native form of the protein, which is 66 kDa.

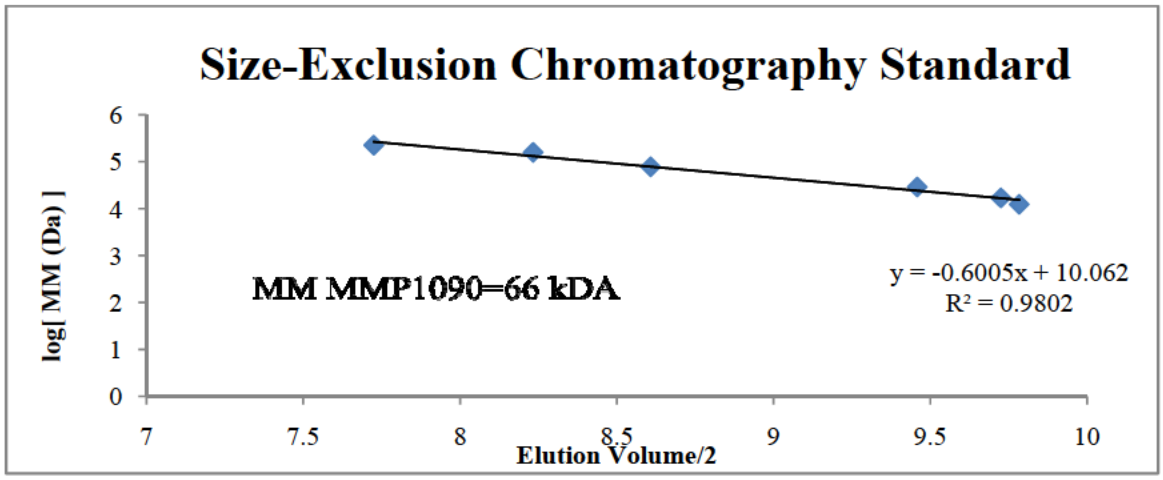

Figure 6: Standard curve of size-exclusion chromatography

Below are various chromatograms of UDP-Glc/UDP-GlcNAc epimerization in the presence and absence of NAD^+. All show about 20% conversion, showing activity with or without NAD^+.

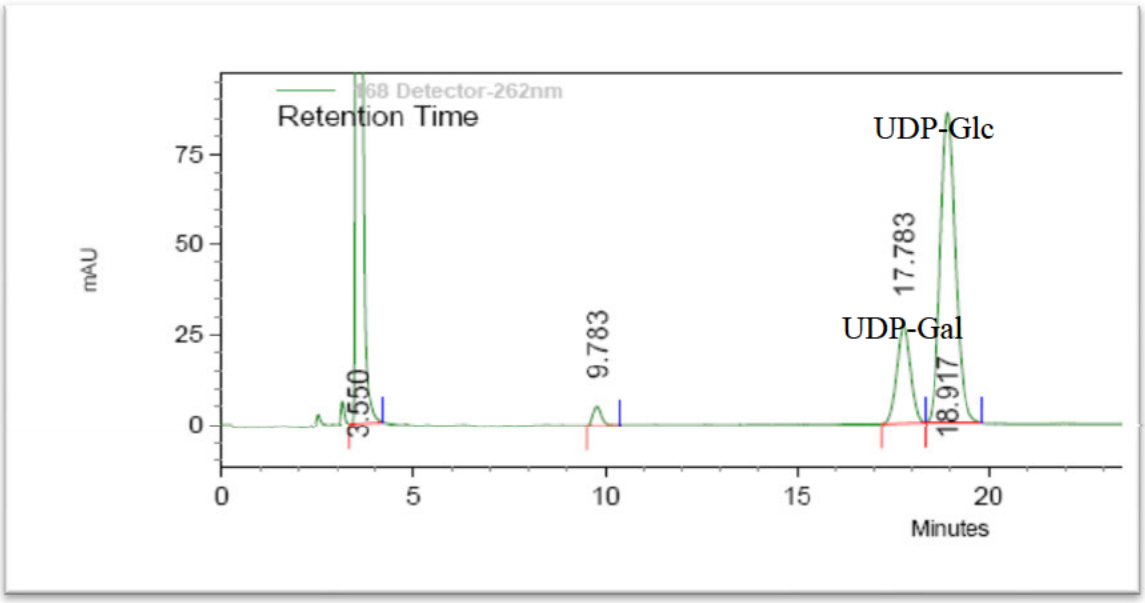

Figure 7: UDP-Glc Epimerization with NAD^+

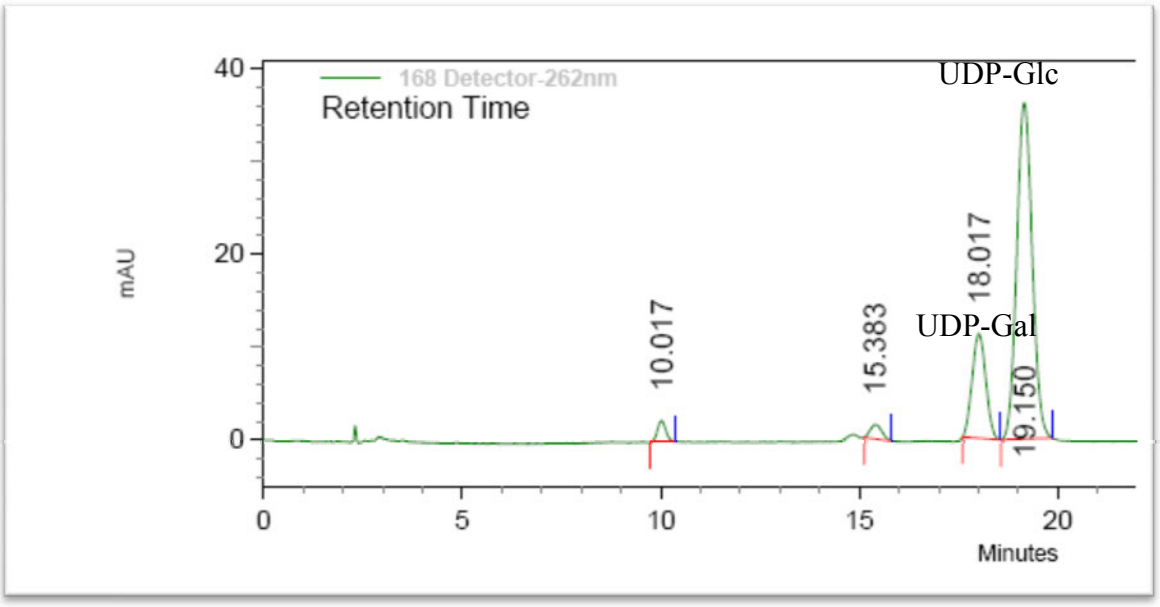

Figure 8: UDP-Glc Epimerization without NAD^+

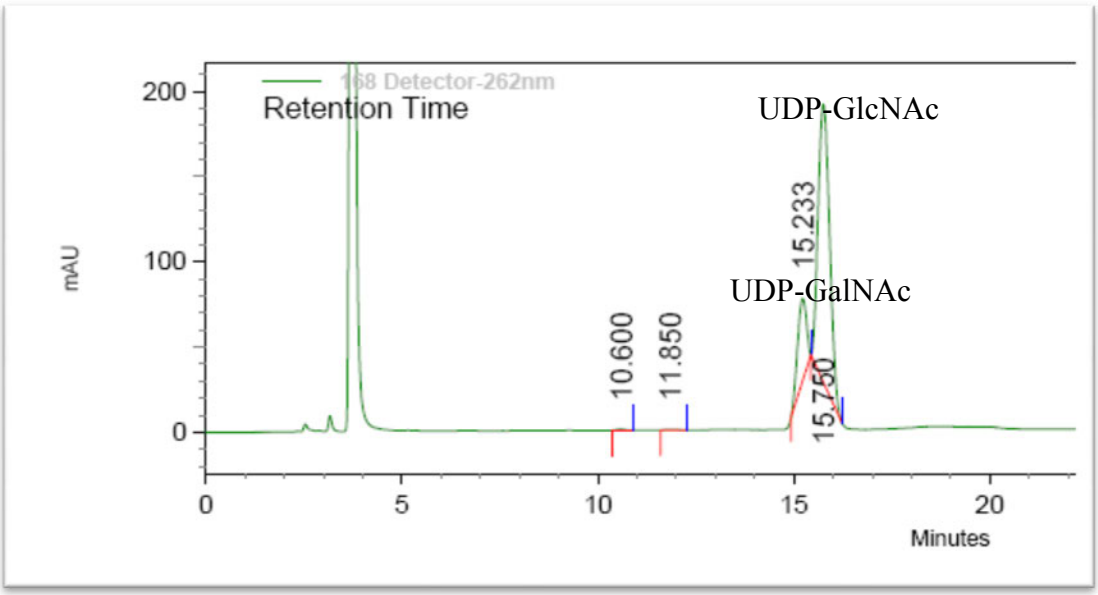

Figure 9: UDP-GlcNAc Epimerization with NAD^+

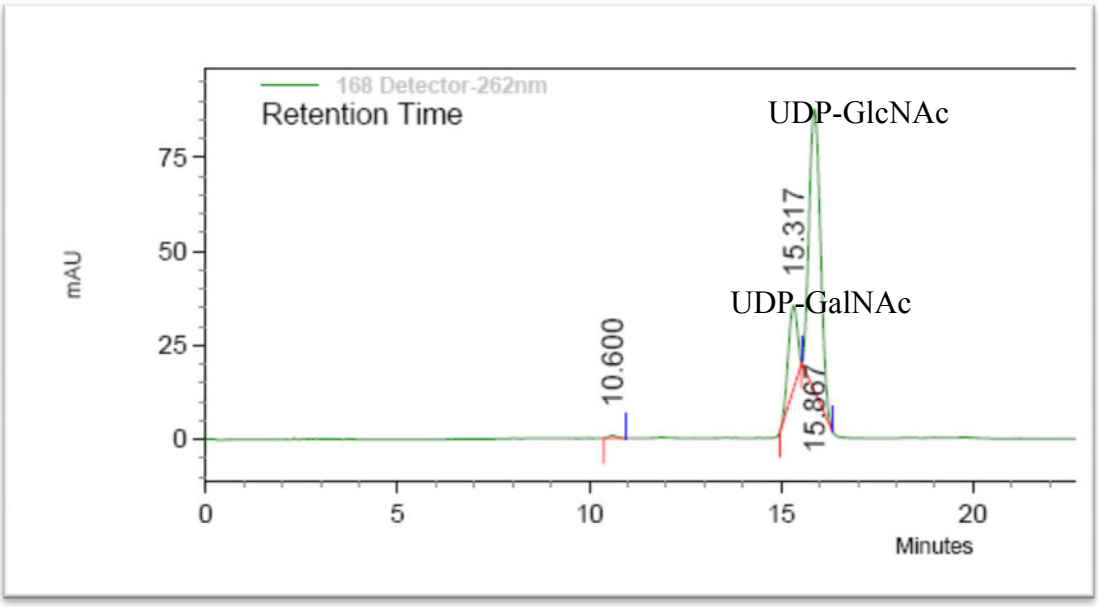

Figure 10: UDP-GlcNAc Epimerization without NAD^+

The next chromatograms are reactions with varying pH buffers. Conversion at pH 8.0 and 8.5 are both approximately 20% but conversion drops to about 7% at pH 9.5.

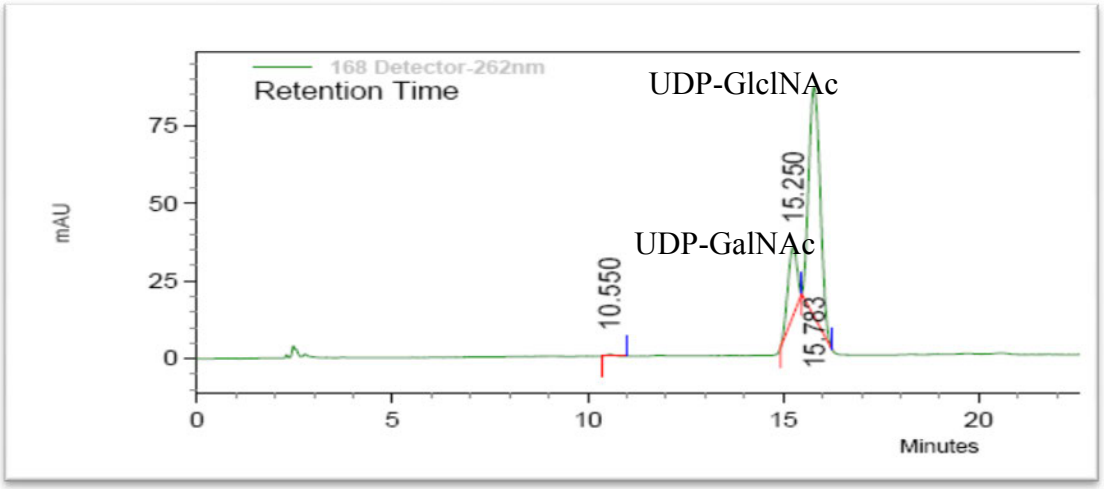

Figure 11: Epimerization of UDP-GlcNAc at pH 8.0

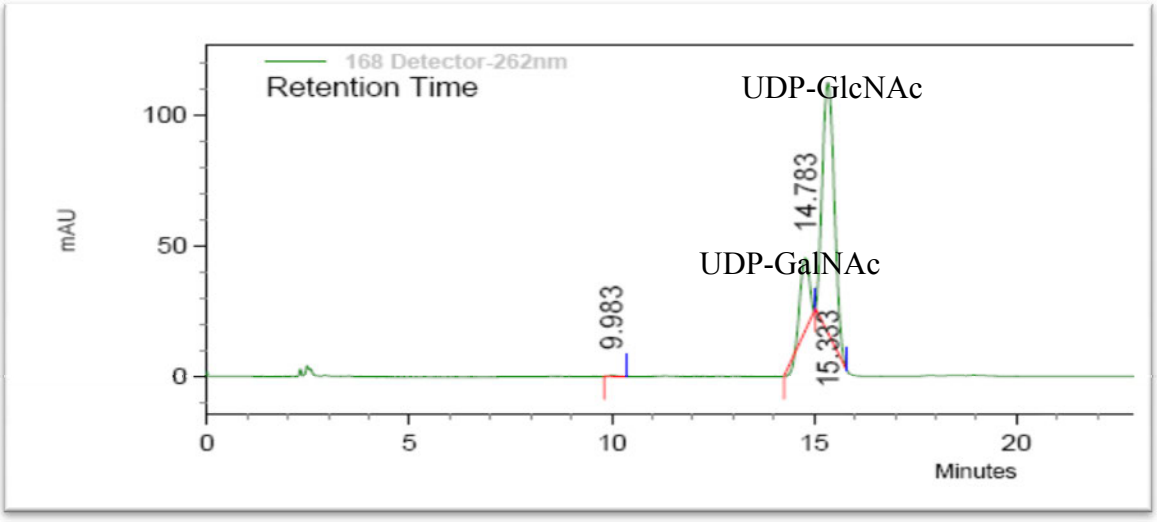

Figure 12: Epimerization of UDP-GlcNAc at pH 8.5

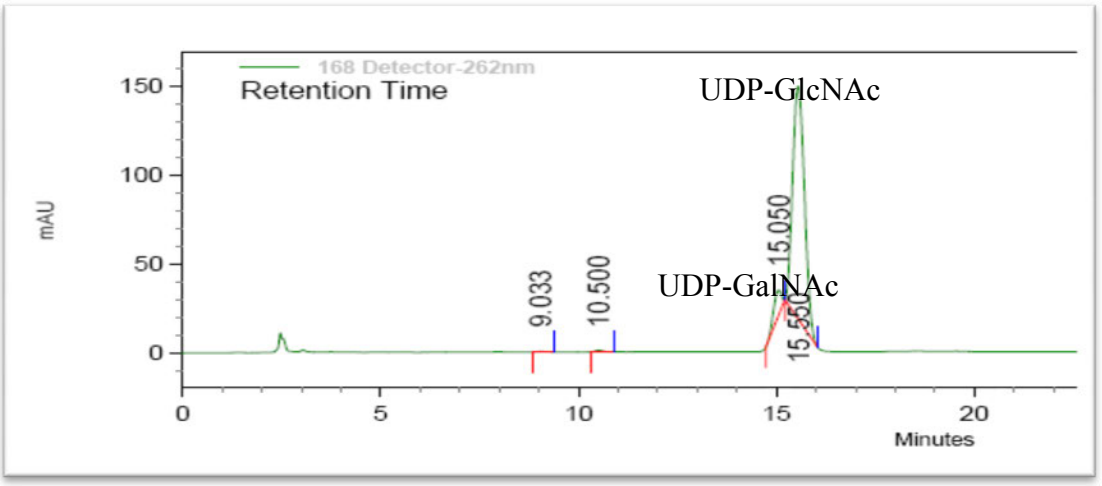

Figure 13: Epimerization of UDP-GlcNAc at pH 9.0

Kinetic Profile of MMP1090

Below are the reaction profiles for the MMP1090 epimerase, with UDP-Gal and UDP-GalNAc as the substrate. Table 1 below summarizes the kinetic profile found through the kinetic experiments, which measured NADH formation through a coupled reaction that combined epimerization by the epimerase and subsequent oxidation by a dehydrogenase.

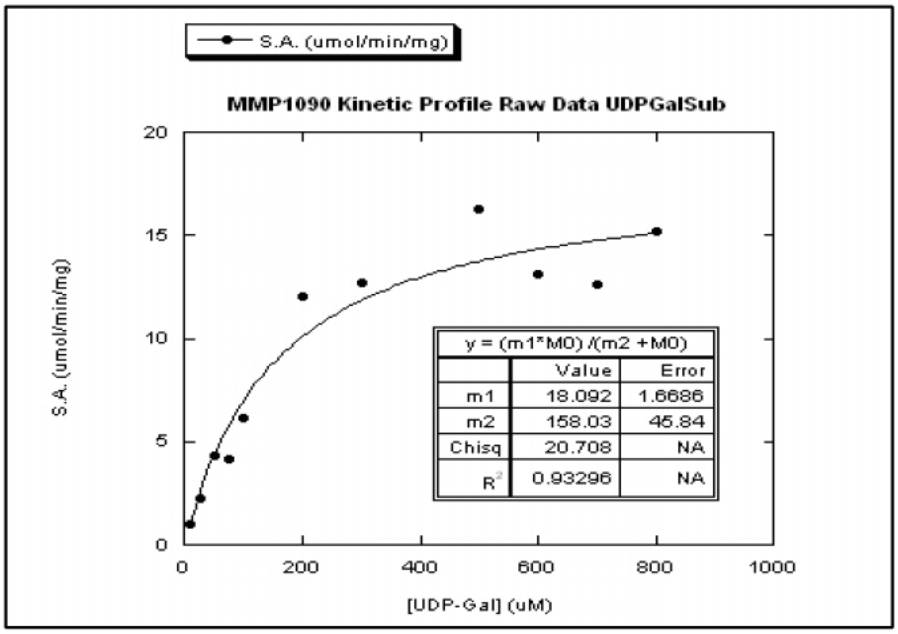

Figure 14: Reaction profile for MMP1090 with UDP-Gal as the substrate.

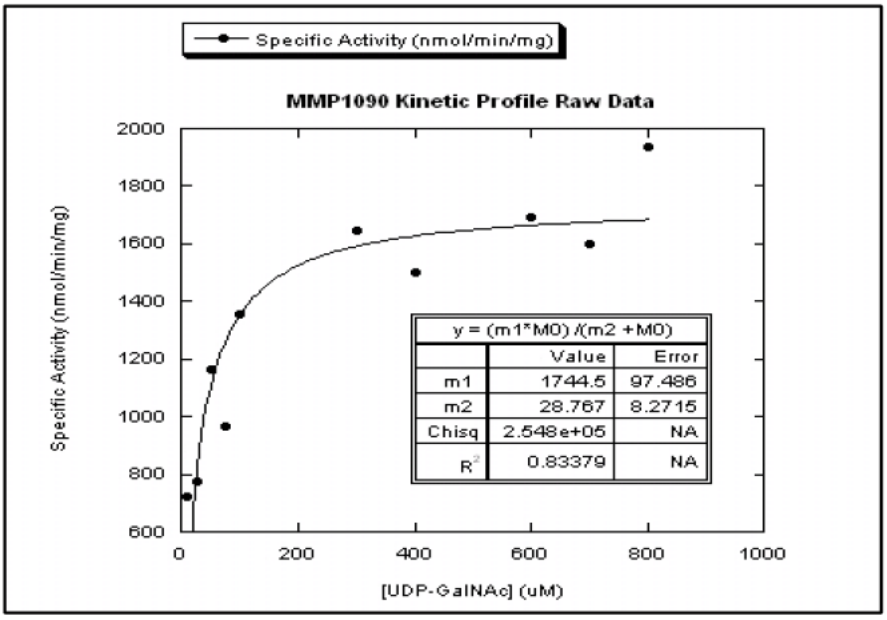

Figure 15: Reaction profile for MMP1090 with UDP-GalNAc as the substrate.

Substrate	K_m (uM)	V_{max} (umol/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}*s^{-1}$)
UDP-GalNAc	29 ± 8.3	1.744 ± .097	0.98	33.7
UDP-Gal	158 ± 45.8	18.092 ± 1.6	10.13	64.1

Table 1: Summary of kinetic profile for MMP1090 epimerase, with UDP-GalNAc or UDP-Gal as the substrate. Comparing their specificity constants (k_{cat}/K_m), it appears the enzyme has a higher specificity for UDP-Gal.

UDP-Gal Assay: Verification of Stoichiometry

The response curve of the verification of stoichiometry for the UDP-Galactose coupled reaction is shown below in Figure 16. The curve indicates an approximate 2 NADH reduced for 1 UDP-Gal epimerized.

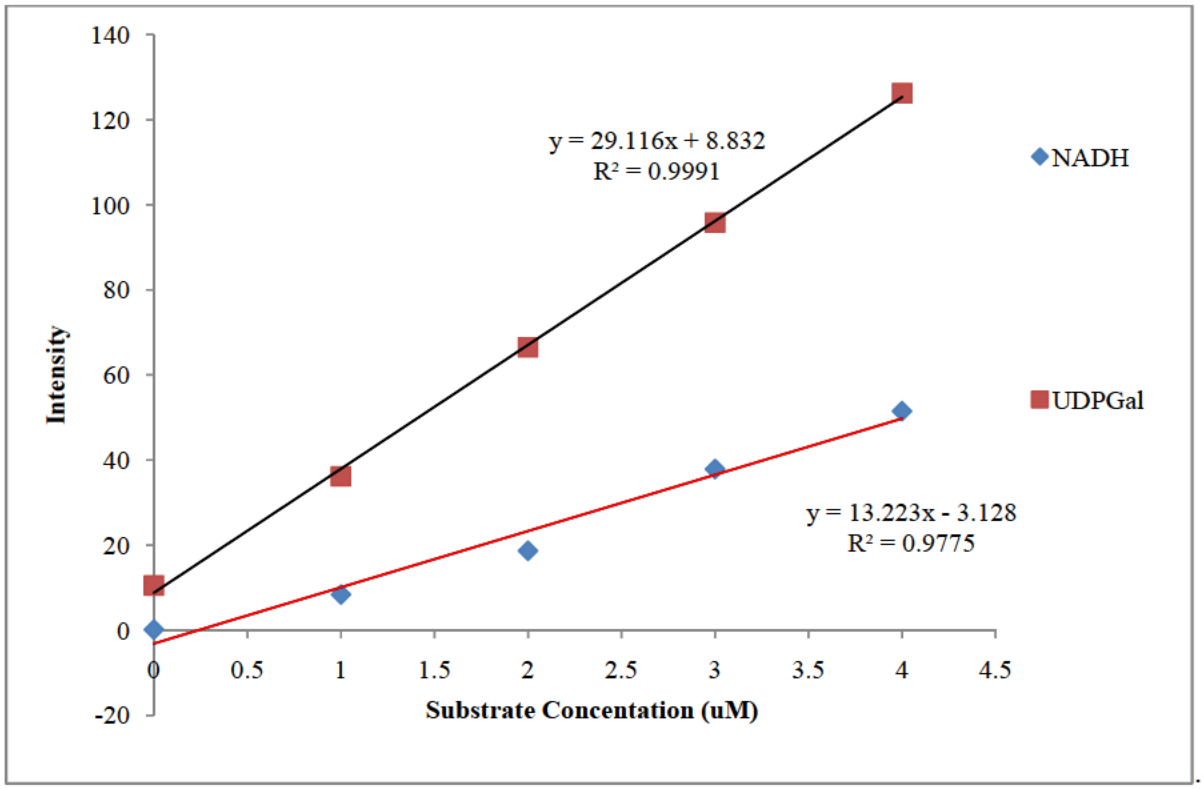

Figure 16: The response curve of stoichiometry by evaluating NADH formation of the coupled reaction with NADH standard and UDP-Gal as the substrate. Comparing the slope, there is an approximate 2.2:1 ratio of UDP-Gal to NADH.

UDP-Gal Assay: Determination of UDP-Gal levels in E. coli

Figure 17 and Figure 18 below shows the standard addition curve for the UDP-Gal+UDP-Glc assay and UDP-Glc Assay respectively. The UDP-Glc concentration of the *E. coli* cell extracts determined from the curves appeared to be 0.211 µM ± 0.0870 while the total UDP-Gal+UDP-Glc concentration appears to be 0.278 µM ± 0.113 µM.

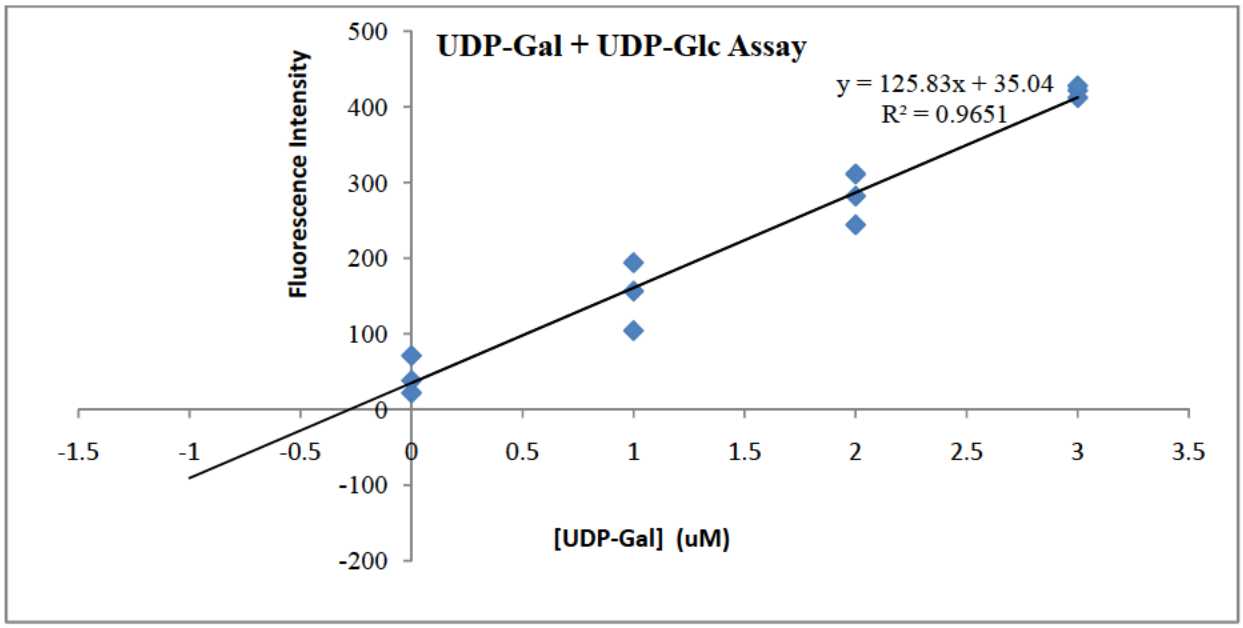

Figure 17: Standard addition curve for the total UDP-Gal+UDP-Glc concentration assay on a sample of *E. coli.*

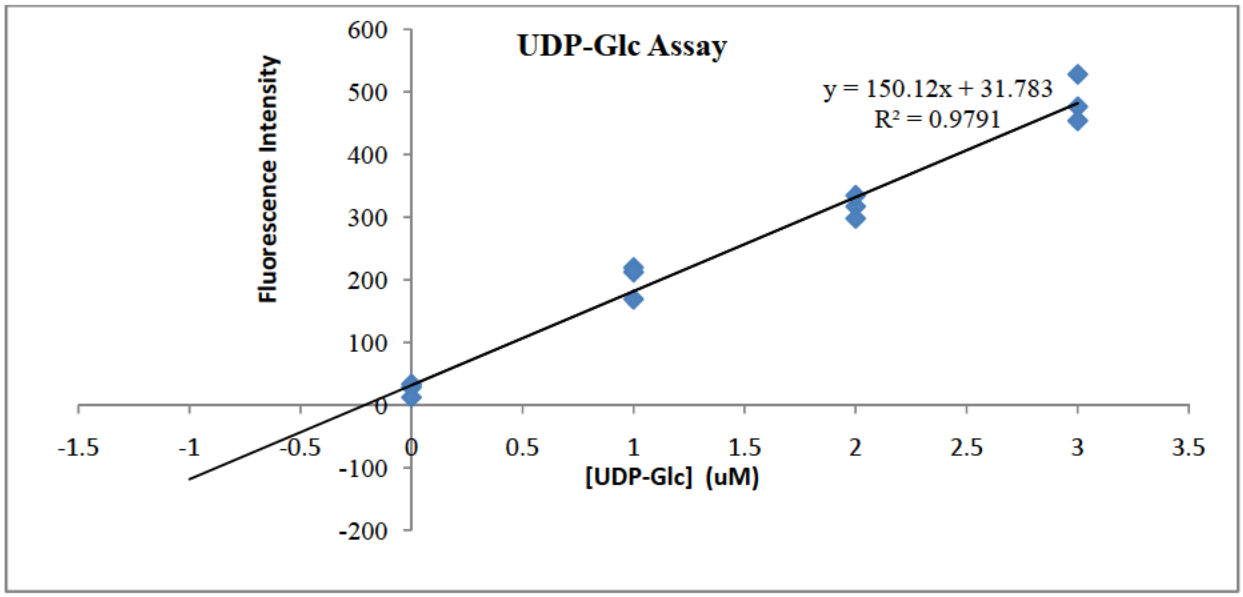

Figure 18: Standard addition curve for the total UDP-Gal+UDP-Glc concentration assay on a sample of *E. coli.*

Finding the difference in the two concentrations values and propagating for error gives a UDP-Gal concentration of 0.0670 µM ± 0.142 µM. Error being far greater than the calculated value gives a high uncertainty on the presence of UDP-Gal in the extracted samples.

Comparing UDP-Glc levels in E. coli: Effects of Glucose-Supplemented Growth Media

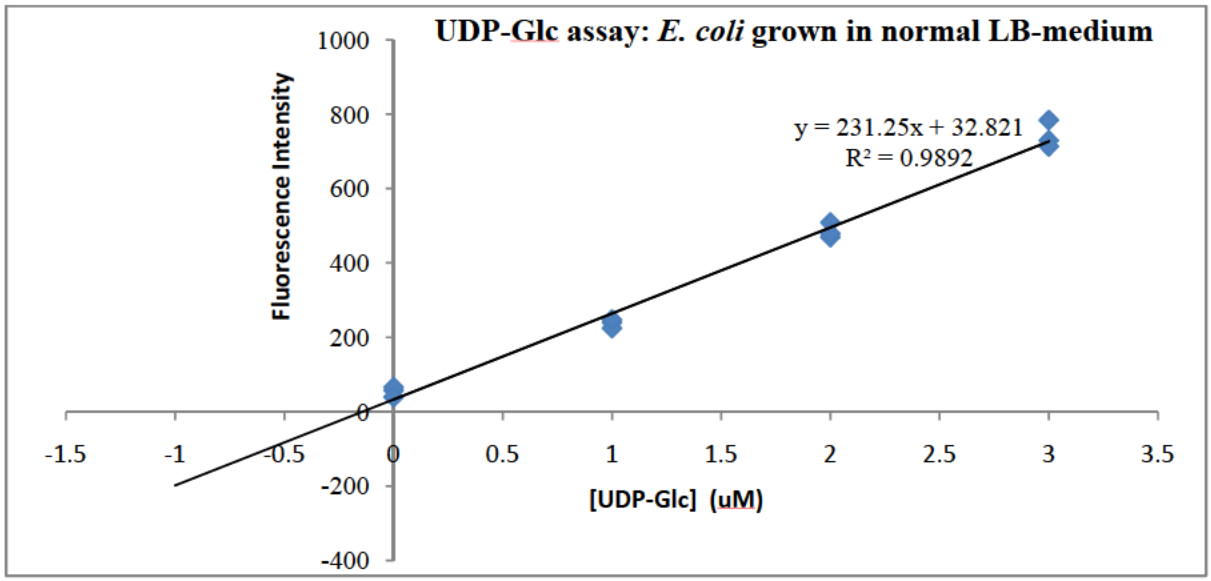

Figure 19: Standard Addition curve of UDP-Glucose Assay for *E. coli* grown in Normal LB-Medium (also used as curve for *E. coli* grown in aerobic conditions).

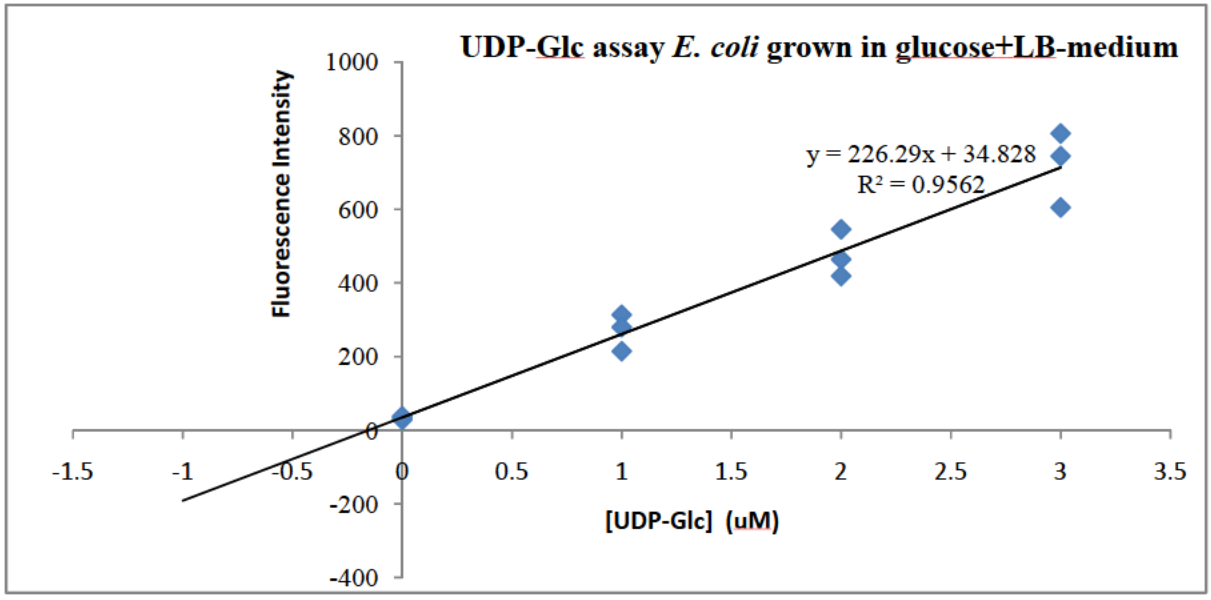

Figure 20: Standard Addition curve of UDP-Glucose Assay for *E. coli* grown in Glucose+ LB-Medium.

Figures 19 and 20 above shows the standard addition curves for the UDP-Glc assay on the two different growth conditions. Table 2 summarizes the results of the measured amount of UDP-Glc per dry mass of *E. coli* of the two experimental conditions. The t-test shows *E. coli* grown in glucose-supplemented LB-media to have significantly higher UDP-Glc levels than *E. coli* grown in normal LB-media.

	Normal LB-Media	**Glucose+ LB Media**
[UDP-Glc] (uM) of 15 µL Cell Extract	0.142 ± 0.0012	0.154 ± .0050
nmol of UDP-Glc/g of cell	4.62 ± .0381	5.40 ± 0.174
t-value	15.0681	
t-table (99% Confidence) **(where d.f. = 2n-4 =6, n=4)**	3.707	

Table 2: Results of the UDP-Glc assay on the *E. coli* cells under Normal and Glucose+ LB-Media. The t-test performed shows a t-value >> t-table, which shows a significant difference between the two values.

Comparing UDP-Glc levels in E. coli: Aerobic vs Semi-Anaerobic Conditions

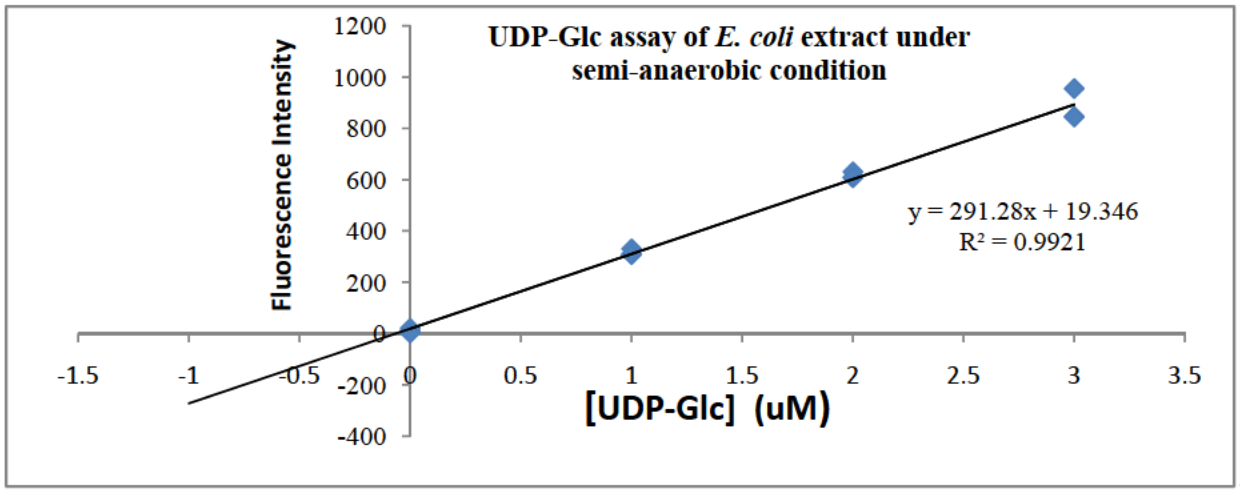

Figure 21: Standard Addition curve of UDP-Glucose Assay for *E. coli* grown under semi-anerobic conditions.

Figure 21 above shows the standard addition curve of the UDP-Glc assay measuring the amount of UDP-Glc in *E. coli* grown under semi-anaerobic conditions. The UDP-Glc assay from Figure 19 was used for comparison of *E. coli* grown in aerobic conditions as vigorous shaking was used to grow the bacteria in the experiment. The results of the aerobic vs. semi-anaerobic experiment are summarized below in Table 3. The t-test shows no significant difference between UDP-Glc levels in *E. coli* grown aerobic and semi-anaerobic conditions.

	Aerobic Conditions	**Semi-Anaerobic Conditions**
[UDP-Glc] (uM) of 15 µL Cell Extract	0.142 ± 0.0012	0.0664 ± 0.00081
nmol of UDP-Glc/g of cell	4.62 ± 0.0381	4.67 ± 0.0566
t-value	2.521	
t-table (99% Confidence) **(where d.f. = 2n-4 =6, n=4)**	3.707	

Table 3: Results of the UDP-Glc assay on the *E. coli* cells under aerobic and semi-anaerobic conditions. The t-test performed shows a t-value < t-table, which shows no significant difference between the two values.

Discussion

Expression and Characterization of MMP1090 Epimerase

The expression of the protein in the soluble phase allowed the protein to be successfully extracted and purified. If it was in the insoluble phase, the enzyme may have resided in the membrane which would have made it harder to purify. The size-exclusion chromatography showed the molecular mass of the native form of the protein to be ~66 kDa, about twice that of the monomeric mass calculated through the ExPASy proteomics tools by using the gene sequence. The native form of the protein having a molecular mass twice that of the monomeric forms suggests that the MMP1090 epimerase is a homodimer, like many other UDP-Gal/UDP-Glc epimerases[6].

Testing for activity of the protein showed that the enzyme is active in epimerizing both UDP-Glc and UDP-GlcNAc. NAD^+ is a possible cofactor in the epimerization reaction[6], and activity without NAD^+ suggests an endogenous NAD^+ cofactor inherently bound to the epimerase. Varying pH showed no significant activity change, although at pH 9.5 there is low conversion, possibly due to degradation of the enzyme.

Kinetic Profile

The kinetic evaluation indicates that the MMP1090 epimerase has a significantly higher specificity for UDP-Gal over UDP-GalNAc, about two-fold when comparing their specificity constants. This could suggest that in *M. maripaludis* UDP-Gal/UDP-Glc epimerization is preferred over UDP-GalNAc/UDP-GlcNAc epimerization. Pathways involving UDP-Gal/UDP-Glc epimerization may occur at higher levels compared to pathways involving the UDP-acetamido sugar epimerization. Table 4 compares the kinetics, using UDP-Gal as the substrate, of the MMP1090 epimerase with other known UDP-Gal epimerases, while Table 5 compares the

kinetics of UDP-GalNAc epimerization. Comparing the apparent K_m, the kinetic parameters determined for the MMP1090 epimerase appears to have comparable values with other UDP-Gal/UDP-Glc and UDP-GlcNAc/UDP-GalNAc epimerases.

Organism	Name of Epimerase	Apparent K_m (uM)	K_{cat} (s^{-1})
M. maripaludis	MMP1090	158	10.13
H. sapiens	GALE [15]	230	-
P. aeruginosa	WbpP [13]	224	-
T. cruzi	TcGALE [14]	114	-

Table 4: Comparison of the MMP1090 with other epimerases using UDP-Gal as the substrate.

Organism	Epimerase name	Apparent K_m (uM)	K_{cat} (s^{-1})
M. maripaludis	MMP1090	29	0.98
P. aeurigosa	WbpP [13]	224	4.52
Sus (Pigs)	*Sus* Epimerase [16]	20	-

Table 5: Comparison of the MMP1090 with other epimerases using UDP-GalNAc as the substrate.

UDP-Gal Assay

The UDP-Gal concentration assays shows promise in determining UDP-Gal levels in cells. However, the assay still needs much optimization. Statistical tests done on initial assay runs show no confidence in the measured concentration values of UDP-Gal. The UDP-Glc + UDP-Gal assay needs to be further optimized and refined to allow for the completion of the UDP-Gal assay. Factors that will be tested will include incubation time, enzyme amount, NAD+

concentration, and pH. Also, experiments will be done more meticulously to reduce the occurrence of user-error. Upon successful creation of the UDP-Gal assay, the assay will be again used to attempt to test UDP-Gal levels in *E. coli* and later perhaps in yeast. The assay can be then used to evaluate factors that may influence UDP-Gal levels in these organisms. Also, the assay may help to evaluate diseases and conditions that involve UDP-Gal. One condition, galactosemia, involves the inability of an organism to metabolize galactose. This condition arises from the disruption of function of any the proteins involved in the galactose metabolism pathway, of which the UDP-Galactose 4-epimerase is one[6]. These other proteins include galactokinase, which phosphorylates galactose, and galactose-1-phosphate uridylyltransferase, which transfers an uridylyl group from UDP-Glc to galactose-1-phosphate to produce glucose-1-phosphate and UDP-Gal[6]. The epimerase functions to regenerate UDP-Glc through epimerization of UDP-Gal[6]. The UDP-Gal assay can be used to compare the differences in UDP-Gal levels in organisms with the disease and without.

Comparing UDP-Glc levels in E. coli:

The UDP-Glc assays performed on *E. coli* cell extracts grown under normal LB-medium and Glucose+LB-Medium showed that cells grown under Glucose+LB-medium had significantly higher levels of UDP-Glc, 5.40 nmol UDP-Glc/g of cell, compared to cells grown under normal LB-medium, 4.62 nmol UDP-Glc/g of cell. This is to be expected as the increased levels of glucose would cause increased sugar metabolism and therefore higher levels of activated UDP-Glc. Replicate experiments will be performed to ensure consistency and reproducibility.

In the comparison of aerobic conditions vs. anaerobic conditions, *E. coli* grown in aerobic conditions appears to have no significant difference in UDP-Glc levels when compared to *E. coli*

grown in semi-anaerobic conditions. Though UDP-Glc levels would be expected to be higher in aerobic conditions due to higher metabolism levels in respiration than fermentation, the cells grown semi-anaerobically were exposed to residual levels of oxygen that probably induced respiration in the cells. As such, glucose metabolism levels may have become comparable to cells grown aerobically. This experiment could be redone with *E. coli* cells grown completely anaerobically to see further investigate the effects of fermentation on UDP-Glc levels. Fermentation would cause a build-up of mixed-acid by-products, which would decrease the intracellular pH of the organism. A separate experiment could investigate the effects of pH on UDP-Glc levels in *E. coli*.

Future Works

Further growth conditions affecting UDP-Glc levels in *E. coli* could be explored. One condition, pH, has been mentioned above. Other factors could include minimal media vs. rich media, and high vs. low temperatures. Experiments would also need to be done to account for the myriad of compounds that are present in the LB-media consisting of yeast extract and casein, an amino-acid source.

As stated before, the UDP-Gal total concentration assay will need to be further optimized to allow for determination of UDP-Gal levels in cells. Once optimized, growth conditions affecting UDP-Gal levels may be explored using the assay just as with the UDP-Glc assay. Also, these UDP-sugar assays may be used to explore sugar metabolism in a variety of other organisms such as S. cerevisiae and even human cells. Since the epimerase also acts on UDP-GalNAc/ UDP-GlcNAc, a UDP-GalNAc total concentration assay will also be attempted to be created to allow for similar applications.

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